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The Selective Proteasome Inhibitors Lactacystin and Epoxomicin Can Be Used to Either Up- or Down-Regulate Antigen Presentation at Nontoxic Doses[1]

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Abstract

The complete inhibition of proteasome activities interferes with the production of most MHC class I peptide ligands as well as with cellular proliferation and survival. In this study we have investigated how partial and selective inhibition of the chymotrypsin-like activity of the proteasome by the proteasome inhibitors lactacystin or epoxomicin would affect Ag presentation. At 0.5–1 μM lactacystin, the presentation of the lymphocytic choriomeningitis virus-derived epitopes NP118 and GP33 and the mouse CMV epitope pp89–168 were reduced and were further diminished in a dose-dependent manner with increasing concentrations. Presentation of the lymphocytic choriomeningitis virus-derived epitope GP276, in contrast, was markedly enhanced at low, but abrogated at higher, concentrations of either lactacystin or epoxomicin. The inhibitor-mediated effects were thus epitope specific and did not correlate with the degradation rates of the involved viral proteins. Although neither apoptosis induction nor interference with cellular proliferation was observed at 0.5–1 μM lactacystin in vivo, this concentration was sufficient to alter the fragmentation of polypeptides by the 20S proteasome in vitro. Our results indicate that partial and selective inhibition of proteasome activity in vivo is a valid approach to modulate Ag presentation, with potential applications for the treatment of autoimmune diseases and the prevention of transplant rejection.

The production of peptide ligands for MHC class I molecules is an intricate cooperative process involving proteases in the cytoplasm or nucleus as well as peptidases in the endoplasmic reticulum (ER)[3] (1). A suitable MHC ligand may be produced in the cytoplasm in its final form, which is subsequently transported into the lumen of the ER via TAP. Alternatively, an N-terminally extended precursor peptide may be generated in the cytoplasm that has the correct class I anchor residue at its C-terminus but requires N-terminal trimming by ER-resident peptidases to fit into the peptide binding groove of an MHC class I molecule (2).

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[3]Abbreviations used in this paper: ER, endoplasmic reticulum; LCMV, lymphocytic choriomeningitis virus; MCA, 7-amido-4-methylcoumarin; MCMV, murine cytomegalovirus; PGPH, peptidylglutamyl-peptide hydrolyzing; Suc, succinyl; (Z), benzyloxycarbonyl; rVV, recombinant vaccinia virus; TFA, trifluoroacetic acid; HAT, hypoxanthine/aminopterin/thymidine.

The protease in charge of generating most of the class I ligands and their precursors in the cytoplasm is the proteasome (3,4). The proteasome consists of a proteolytic core complex called 20S proteasome and a number of regulatory complexes that associate with the 20S core to control its activity and recruit and unfold substrate proteins. The 20S proteasome is shaped like a cylinder constituted of four stacked rings (5). The outer two rings are made up of seven α-type subunits that mediate the association with the regulatory complexes, whereas the inner two rings consist of seven subunits of the β-type. Three of the β-subunits, designated δ, MB-1, and Z, possess in their processed form N-terminal threonine residues that contribute their γ-hydroxy groups as nucleophiles to the three peptidolytically active centers of the protesaome. The catalytic activities of the proteasome have been classified with fluorogenic peptides as chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-peptide hydrolyzing (PGPH) (4). Mutagenesis experiments in combination with inhibitor studies suggested an assignment of PGPH activity to the δ subunit, of trypsin-like activity to the δ subunit, and of chymotrypsin-like activity to the subunit MB-1 (6–9).

The central involvement of the proteasome in MHC class I-restricted Ag processing was established with the help of proteasome-specific inhibitors. These inhibitors prevented the peptide-dependent maturation of MHC class I molecules in the ER and interfered in the most cases with class I-restricted presentation of T cell epitopes (10–14). In some models, however, treatment with proteasome inhibitors enhanced, rather than prevented, presentation of the respective epitopes. It was proposed that in these cases the epitopes or their precursor were generated by proteases other than the proteasome and that the proteasome destroyed, rather than generated, the respective peptides (15–17). Proteases such as the tripeptidyl peptidase II (18), furin (19), or the thimet oligopeptidase (20) have been suggested to play a role in the production of MHC class I ligands, although the in vivo evidence that these proteases are critically involved in Ag processing is either missing or scarce. There is, however, an alternative explanation for the aforementioned phenomenon; namely, that proteasome inhibition was only partial and that the residual proteasome activities produced different peptide fragments that could be better suited as MHC class I ligands or their precursors. Evidence for this scenario was recently obtained by Valmori et al. (17), who found that selective proteasome inhibition both in vivo and in vitro resulted in the generation of a MAGE-3 epitope that was not generated in the absence of proteasome inhibition. These findings suggest that partial proteasome inhibition may be a means to either prevent or enable Ag presentation. However, the dosage of proteasome inhibitors applied in these experiments was too high to allow cellular proliferation or survival.

In the present study we set out to test whether a partial inhibition of the proteasome by treating cells with nontoxic doses of selective proteasome inhibitors could be employed to either up- or down-regulate Ag processing. For this investigation we chose lactacystin and epoxomicin as selective proteasome inhibitors because they both inhibit the chymotrypsin-like activity of the proteasome much faster than the trypsin-like and PGPH activities. Lactacystin is a *Streptomyces* metabolite that was discovered by virtue of its ability to induce cell cycle arrest and neurite outgrowth in a neuroblastoma line (21). The molecular target of lactacystin was later found to be the proteasome and, in particular, the N-terminal threonine residue of the β-type proteasome subunits MB-1 and, to a lesser extent, the subunit Z (5,22). The α′,β′-epoxyketone inhibitor epoxomicin is a product of an *Actinomycetes* strain that was identified based on its ability to inhibit melanoma growth in vivo (23). Only very recently was the proteasome identified as the target of epoxomycin that also covalently binds to the subunits MB-1 and Z (24). Although the proteasome inhibition profiles of these two inhibitors are very similar, epoxomicin is about 100-fold more potent than lactacystin.

For our study we selected four immunodominant viral epitopes that have been well characterized with respect to their generation and presentation. Three of these epitopes are

derived from the lymphocytic choriomeningitis virus (LCMV) (25). The H-2L^d restricted epitope NP118 is processed from the LCMV nucleoprotein (residues 118–126), whereas the H-2D$_b$-restricted epitopes GP33 and GP276 are derived from residues 33–41 and 276–286 of the LCMV glycoprotein, respectively. In addition, we analyzed the H-2L^d-restricted epitope pp89–168 of murine CMV (MCMV) encompassing residues 168–176 of the MCMV immediate early protein pp89 (26). In this analysis we found that treatment of cells with low concentrations of lactacystin or epoxomicin enhanced the presentation of one epitope (GP276), whereas others were reduced (GP33, NP118, pp89–168). At higher inhibitor concentrations, which interfered with cellular proliferation and survival, the presentation of all four epitopes was abolished, suggesting that their generation was proteasome dependent. These results are convincing evidence that a partial and selective inhibition of the proteasome at nontoxic doses can be applied to modulate Ag presentation to either enhance or attenuate an ongoing cytotoxic immune response.

Materials and Methods

Cells and media

C4 is a murine fibroblast line derived from embryonic BALB/c mice by SV40 infection in vitro (H. Hengel and U. H. Koszinowski, unpublished data). Clone B8 was derived from C4 cells by transfection of the immediate early gene 1 of MCMV encoding the pp89 protein (27). MC57 is a C57BL/ 6-derived methylcholanthrene-induced fibrosarcoma cell line (28). MCGP is a MC57-derived cell line expressing the LCMV glycoprotein (29). RMA-S is a TAP1-deficient variant of the Rauscher virus-transformed mouse T cell line RMA (H-2^b) (30). The human T cell leukemia Jurkat and the MHC class II-deficient human lymphoblastoid line T2 were obtained from American Type Culture Collection (Manassas, VA). T2-L^d was obtained by transfection of T2 with an H-2L^d expression construct. The BWZ.36.1/CD8α fusion partner had been made by transfecting $\alpha^-\beta^-$-BW5147 mouse lymphoma cells with NF-AT-*lacZ* reporter and CD8α expression constructs as described previously (31). All cells were grown in complete IMDM containing 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/ streptomycin; supplements were required for B8 (250 μg/ml G418), MCGP (0.8 mg/ml G418), T2-L^d (1 mg/ml G418), BWZ.36.1/ CD8α (0.5 mg/ml hygromycin B, 0.5 mg/ml G 418), GP33Hyb, GP276Hyb, NP118Hyb, and pp89Hyb (1× HAT, 0.5 mg/ml hygromycin B).

Viruses

The LCMV-WE strain was originally obtained from F. Lehmann-Grube (Hamburg, Germany) (32). The virus was grown and titrated on L929 cells, and virus stocks were stored at −70°C. Recombinant vaccinia virus (rVV) expressing the pp89 protein (33) was raised and titrated on BSC40 cells and stored at −70°C.

Mice

BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from the Institut für Labortierkunde, Tierspital Zurich (Zurich, Switzerland) and kept in a specific pathogen-free environment.

Peptides

Synthetic peptides were obtained from Echaz Microcollections (Tubingen, Germany) and represent either the LCMV-WE glycoprotein-derived T cell epitopes GP33–41 (KAVYNFATC; GP33) and GP276–286 SGVENPGG YCL (GP276) restricted by H-2D^b or the LCMV-NP-derived epitope NP118–126 RPQASGVYM (NP118) and the MCMV-pp89 epitope pp89/168–176 (YPHFMPTNL; pp89–168) presented on H-2L^d. The 25-mer peptides used for proteasome digests encompassed LCMV-WE nucleoprotein residues 108–132 KLKAKIMRTERPQASGVYMGNLTAQ, LCMV-WE glycoprotein residues 271–295

TLSDSSGVENPGGYCLTKWMILAAE, and MCMV-pp89 residues 162–186 RLMYDMYPHFMPTNLGPSEKRVWMS.

Antibodies

KL 25 is a mouse mAb reactive with the LCMV glycoprotein (34), and VL-4 is a rat mAb reactive with the LCMV nucleoprotein (35).

Purification of 20S proteasome and fluorogenic peptide assays

The purification and quantitation of the 20S proteasome from B8 cells as well as fluorogenic peptide assays were performed exactly as previously described (27). For the titration of lactacystin the inhibitor was added at the same time as the substrate, and fluorescence of the MCA and β-naphthylamide leaving groups was measured after 30, 60, and 90 min to ensure that the reaction proceeded in a linear fashion. Lactacystin was purchased from Biomol (Plymouth Meeting, PA), and epoxomicin was synthesized as previously described (36).

Generation of T cell hybridomas

CTL lines recognizing LCMV or MCMV epitopes were derived by infecting mice i.v. either with 200 PFU of LCMV-WE or 5×10^6 PFU of rVV-pp89 and preparing a spleen cell suspension not earlier than 1 mo after infection. Spleen cells were plated at 4×10^6 cells/well in a 24-well plate in IMDM, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5×10^{-5} M 2-ME, 10% rat Con A supernatant (as a source of IL2). Cells were restimulated every 7–10 days with peptide-loaded and irradiated (80 Gy) RMA-S or T2-L^d cells at a CTL to APC ratio of 5:1. Loading with synthetic peptides occurs for 1 h at room temperature with 10^{-7} M of the respective peptide. The cells were $CD8^+$ and of single specificity after three rounds of restimulation. CTLs were harvested 3–4 days poststimulation, and 5×10^6 cells were mixed with equal numbers of BWZ36.1/CD8α fusion partner in a 50-ml Falcon tube. Cells were washed in 37°C warm serum-free IMDM and centrifuged at $500 \times g$ for 5 min. The supernatant was removed, and the pellet was loosened by gentle tapping. One milliliter of prewarmed 50% PEG 1500 (w/v) solution in 75 mM HEPES (pH 8.0) was slowly added over the course of 1 min, with gentle stirring with the pipette tip in between drops. After gently stirring for 1 additional min the polyethylene glycol was diluted by addition of 2 ml of warm serum-free medium added in single drops over the course of 2 min. An additional 7 ml of warm serum-free medium was added over 2 min before the tubes were placed in a 37°C water bath for 8 min. The cells were centrifuged at 1400 rpm for 5 min and gently resuspended in prewarmed complete IMDM. The cells ($1–3 \times 10^4$ in 0.1 ml/well) were plated in 96-well flat-bottom plates. After a 24-h incubation period 50 μl of 3 × HAT (300 μM hypoxanthine, 1.2 μM aminopterin, and 48 μM thymidine; prepared from 50× frozen stock (Sigma, Buchs, Switzerland)) in complete IMDM supplemented with 1.5 mg/ml hygromycin B were added. CTL hybrids were generally observed within 2 wk of culture. Individual clones were expanded into 24-well plates and tested for specificity in *lacZ* assays. Positive clones were subcloned by limiting dilution one to three times, and the monoclonal lines were maintained in complete IMDM and 0.5 mg/ml hygromycin B/1× HAT. The most stable hybridomas were selected for further application and named according to their specificities GP33Hyb, GP276Hyb, NP118Hyb, and pp89Hyb.

T cell stimulation assays

MC57 or B8 mouse fibroblast cells were infected with LCMV-WE with a multiplicity of infection of 0.05, and after 24 h the cell surface expression of the LCMV glycoprotein was confirmed by flow cytometric analysis using the KL25 mAb. An acid wash procedure was performed to remove pre-existing peptide/MHC complexes from the surface as previously described (11). Briefly, about 10^7 cells were resuspended in 1 ml of buffer A (131 mM citric

acid and 66 mM disodium phosphate, pH 3.1) and incubated under mild agitation for 2 min at 25°C and centrifuged for 1 min. The supernatant was immediately removed, and the cells were resuspended in 10 ml of complete IMDM medium and washed once in complete medium. Cells were then plated in 96-well round-bottom plates in 100 μl of complete medium, and lactacystin or epoxomicin was added at the indicated concentration. After 5 h (for B8 cells) or 12 h (for MC57 cells) of incubation at 37°C in 5% CO_2 the cells were washed once with PBS to remove lactacystin, and CTL hybridomas (5×10^4) in a volume of 100 μl were added and incubated again for 18 h. Cells were spun down, and after a single wash with PBS the induced *lacZ* activity in the pelleted cells was measured in a chromogenic *lacZ* assay using chlorophenol red β-galacto-side (Roche, Basel, Switzerland) as substrate. The cells were lysed by addition of 100 μl of Z buffer (0.15 mM chlorophenol red β-galactoside, 100 mM 2-ME, 9 mM $MgCl_2$, and 0.125% Nonidet P-40 in PBS), and after a 1- to 4-h incubation at 37°C the absorbance at 570 nm (reference wave-length at 620 nm) was read using a SpectraFluor Plus plate reader (Tecan, Groding/Salzburg, Austria). For external loading of cells with synthetic peptides as a positive control, 5×10^4 cells were plated in 96-well round-bottom plates, and after a single wash with PBS, peptides were added at a final concentration of 10^{-7} M in a final volume of 100 μl. After an incubation for 1 h at room temperature the cells were washed once with PBS before addition of hybridomas.

Metabolic labeling and immunoprecipitation

Cells were infected for 24 h with LCMV (multiplicity of infection, 0.05), and 2×10^6 confluent cells were starved in cysteine/methionine-free RPMI 1640 plus 10% dialyzed FCS for 45 min at 37°C and labeled with 0.1 mCi/ml Tran ^{35}S label (ICN, Eschwege, Germany) for 1.5 h. Labeling medium was removed, and cells were washed with PBS and chased in complete IMDM medium for the indicated time periods. Cells were harvested and lysed for 45 min on ice in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2% Nonidet P40, 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, and 0.85 mM PMSF). The postnuclear lysates were counted for [^{35}S]methionine/cysteine incorporation, and equal aliquots were used for immunoprecipitation. The lysate was precleared for 1 h at 4°C with protein A or G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) followed by immunoprecipitation with the indicated Ab bound to protein G-Sepharose for 3 h at 4°C. The precipitates were washed three times in NET-TON (650 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 1 mg/ml OVA, and 0.05% NaN_3), and after a change of tubes three times with NET-T (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, and 0.05% NaN_3). The beads were boiled in reduced Laemmli sample buffer, and 10% SDS-PAGE was performed before analysis of radioactivity on dried gels using a BAS 1500 radioimager (Fuji, Tokyo, Japan).

Western blotting

MC57 cells were treated for 16 h with the indicated concentrations of lactacystin, and cells were lysed in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, and 0.85 mM PMSF for 30 min at 4°C. The postnuclear supernatant was quantified by optical density, and aliquots of 130 μg of protein were applied to a 7.5% SDS-PAGE. The gels were blotted onto 0.2 μM nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), blocked with PBS/10% horse serum/5% (w/v) low fat dry milk/0.4% Tween-20, and agitated overnight at 4°C with a polyclonal anti-ubiquitin rabbit Ab (Dako, Zug, Switzerland) in PBS/2% low fat dry milk/ 0.1% Tween 20. The blots were washed and incubated for 1 h with the HRP-conjugated secondary Ab. After extensive washing with PBS/0.4% Tween 20, proteins were visualized on x-ray films using chemiluminescence substrate (Roche).

Proteasomal fragmentation of polypeptides and analysis of peptide products by HPLC and Edman degradation

Eighty micrograms of a synthetic 25-mer peptide derived from the sequence of the LCMV-nucleoprotein (residues 108–132), LCMV glyco-protein (residues 271–295), or MCMV-pp89 (residues 162–186) was incubated with 4 μg of purified 20S proteasome in a total volume of 1200 μl of digestion buffer (30 mM Tris-HCl (pH 7.5), 10 mM KCl, and 2 mM DTT) at 37° C in the presence or the absence of lactacystin. At the indicated time points aliquots of 300 μl were taken and frozen to stop the reaction. These cleavage products were analyzed on a μRPC C2/C18 SC 2.1/10 reverse phase column using a SMART System (Pharmacia). Eluent A was 0.1% trifluoroacetic acid (TFA); eluent B was 70% acetonitrile and 0.1% TFA. The gradient was 10–30% eluent B in 55 min; the flow rate was 100 μl/min. Peak fractions were collected, dried, and redissolved in 60% acetonitrile/0.1% TFA. For the identification of peptides, the samples were coimmunoprecipitated with a matrix of α-cyano-4-hydroxycinnamic acid in acetone and analyzed by MALDI-MS (VG-TofSpec, Fison Instruments, Manchester, U.K.). For microsequence analyses of the HPLC-separated peptide samples, a Procise protein sequencer system (Applied Biosystems, Foster City, CA) was used.

Apoptosis assay

RMA cells were treated with lactacystin at the indicated concentrations for 14 h. As a positive control 2×10^6 Jurkat cells in 2 ml of complete medium were incubated with 200 ng of anti-human FAS mAb 7C11 (Immunotech, Marseilles, France) for 6 h. Cells were washed in PBS once, and staining with annexin V-FITC (Clontech, Palo Alto, CA) was performed for 20 min according to the manufacturer's instruction. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, Basel, Switzerland).

Thymidine incorporation

A number of 4×10^4 RMA or MC57 cells were plated in triplicate in round-bottom 96-well plates in 200 μl of medium supplemented with the indicated concentrations of lactacystin. The lactacystin containing growth medium was exchanged every day to avoid premature inactivation. [^{3}H]Thymidine was added to different wells at a final concentration of 2.5 μCi after 0, 24, 48, or 72 h, and cells were incubated for 16 h and subsequently harvested by a Multimesh 2000 harvester (Dynatech, Embrach) onto glass microfiber filters, and the dried filters were covered by liquid scintillation fluid and counted in a beta counter.

Results

Effect of lactacystin on peptide hydrolysis by the proteasome in vitro

To find appropriate concentrations for a selective and partial inhibition of the chymotrypsin-like activity of the 20S proteasome, we isolated 20S proteasomes from B8 fibroblasts to apparent homogeneity and measured the effect of titrated concentrations of lactacystin on the in vitro hydrolysis of four fluorogenic peptides. As displayed in Fig. 1, the substrates Suc-LLVY-MCA and (Z)-GGL-MCA, which are frequently used to monitor chymotrypsin-like activity, were inhibited at 1 μM lactacystin to 66 and 79%, respectively. At the same concentration the trypsin-like activity measured with the substrate Bz-VGR-MCA was not affected, and the PGPH activity determined with the (Z)-LLE-β-naphthylamide substrate was slightly enhanced. It is noteworthy that the PGPH activity increased to a maximum of 2-fold of the original activity, while the other three activities were increasingly inhibited with rising lactacystin concentrations. This may reflect the cooperativity between the chymotrypsin-like and the PGPH activity, which has to date only been shown with different substrates for the respective active sites but not with the employment of a selective inhibitor (37). Taken together

we concluded from these titrations that a concentration range of 0.1–1 μM lactacystin would be an appropriate starting point to determine potential in vivo effects on Ag presentation.

Generation and characterization of T cell hybridomas for the analysis of Ag presentation

A frequent problem with CTL lines generated by in vitro restimulation is that some CTL lines cannot be easily maintained for very long periods and may vary in their affinity and cytotoxic activity over the period of restimulations. To facilitate a thorough analysis of Ag processing we therefore generated T cell hybridomas from CTLs obtained from mice infected with LCMV-WE or a rVV encoding the pp89 protein of MCMV (rVV-pp89). After three weekly in vitro restimulations with synthetic peptides corresponding to the epitopes LCMV-GP33, LCMV-GP276, LCMV-NP118, and MCMVpp89–168, respectively, the CTLs were fused with BWZ36.1/CD8α cells. This fusion partner has the advantage that it expresses cDNAs for CD8α and the bacterial β-galactosidase (*lacZ*) reporter gene under transcriptional control of the IL-2 promotor and enhancer. Antigenic stimulation of the generated T cell hybridomas can thus be easily monitored in chromogenic *lacZ* assays. The specificity analysis of four selected hybridomas designated GP33Hyb, GP276Hyb, NP118Hyb, and pp89Hyb revealed that they possessed the expected peptide specificity and that the response was restricted by the appropriate MHC class I molecule (Fig. 2). Also, the recognition of endogenously processed Ags was as expected, as the LCMV glycoprotein transfectant MCGP was only recognized by the GP33- and GP276-specific hybridomas, and the MCMV pp89 transfectant B8 exclusively stimulated the pp89–168 hybridoma. Interestingly, a comparison of the sensitivity of the hybridomas and the parental CTL lines revealed that the hybridomas were 10- to 100-fold less sensitive for the respective peptide epitopes (38). This can be a major advantage for the analysis of Ag presentation, as the sensitivity of CTLs in chromium release assays may be too high to detect subtle differences in Ag processing.

Low concentrations of lactacystin can either enhance or reduce Ag presentation

To determine the effect of titrated amounts of lactacystin on Ag presentation in the LCMV model the fibroblast line MC57 (H-2^b) or B8 (H-2^d) was infected with LCMV for 24 h. Subsequently, the cells were treated with a mild acid wash procedure to remove pre-existing epitopes from class I molecules, and the cells were incubated for 5 or 12 h with the indicated concentrations of lactacystin before stimulation of the hybridomas specific for GP33/ H-$2D^b$, GP276/H-$2D^b$, and NP118/H-$2L^d$ was determined in *lacZ* assays. To monitor presentation of the MCMV pp89 epitope we used the uninfected B8 fibroblast cells, because they constitutively express the pp89 protein. As shown in Fig. 3*A*, the presentation of the GP33, NP118, and pp89 epitopes was reduced in a dose-dependent manner in the range from 0.5 to 10 μM lactacystin. Already at 0.5 and 1 μM lactacystin a substantial reduction in the presentation of these three epitopes was observed in three independent experiments. The presentation of the LCMV-GP276 epitope, in contrast, was enhanced by treatment with 0.5 μM lactacystin by about 40%. Nevertheless, the generation of this epitope seemed to be proteasome dependent, as its presentation was reduced to background levels at 10 μM lactacystin. A titration of lactacystin concentrations below 0.5 μM demonstrated that GP276 presentation was gradually enhanced from 0.03 μM until a maximum was reached at 0.5 μM (Fig. 3*B*), and this increase correlated with a proportional increase in the inhibition of the chymotrypsin-like activity of the proteasome (Fig. 1). To exclude that the inhibition of epitope presentation at higher lactacystin concentrations was due to a reduction in LCMV replication or the production of LCMV gene products, we performed immunoprecipitations of the LCMV glycoprotein from infected cells treated with lactacystin at different concentrations for 24 h. As shown in Fig. 3*C*, no influence of lactacystin on the production of LCMV glycoprotein was detectable, and this result was confirmed with flow cytometric analysis of LCMV glycoprotein surface expression and intracellular nucleoprotein expression (data not shown). Taken together, our results suggest that low concentrations of lactacystin can be used to markedly up- or down-regulate Ag

presentation. This effect was dependent on the nature of the epitope rather than on the synthesis rate or half-life of the antigenic protein, as the presentation of two epitopes originating from the same protein (GP33 and GP276) were affected in opposite ways.

The proteasome inhibitors lactacystin and epoxomicin similarly affect proteasomal peptide hydrolysis and Ag presentation

To further substantiate that the observed effects on Ag presentation were due to a partial inhibition of the chymotrypsin-like activity of the proteasome, we used the specific proteasome inhibitor epoxomicin, which resembled lactacystin in that it also inhibited the chymotrypsin-like activity of the proteasome much faster than the other peptidolytic activities (Fig. 4A) (24). Interestingly, epoxomicin also led to a reduction of LCMV GP33 presentation and an enhancement of GP276 presentation at concentrations at which the chymotrypsin-like activity of the proteasome was partially inhibited, whereas the generation of both epitopes was prevented at higher concentrations. Compared with lactacystin, however, the epoxomicin-mediated effects on both peptide hydrolysis and Ag presentation were observed at 100- to 1000-fold lower concentrations due to the higher potency of the latter inhibitor (24). This confirmation of our results with a second, very specific proteasome inhibitor make it very unlikely that the cellular phenomena are due to the inhibition of proteases other than the proteasome.

Effect of lactacystin on the degradation of LCMV glycoprotein, nucleoprotein, and ubiquitin conjugates

To compare the effects of proteasome inhibition on Ag presentation and on the half-life of the proteins from which the respective epitopes are generated we performed pulse-chase experiments with LCMV-infected MC57 and B8 fibroblasts. The LCMV glycoprotein is synthesized with a 58-aa-long ER leader that is cleaved after cotranslational insertion of the proprecursor protein into the ER lumen (39). Subsequent to glycosylation, the transmembrane 70-kDa glycoprotein precursor GP-C is cleaved again in a late secretory compartment to yield the transmembrane 35-kDa protein GP2 and the 44-kDa noncovalently attached ER lumenal protein GP1. GP1 and GP2 then assemble into tetrameric complexes, which migrate to the cell surface (40). The GP33 epitope is derived from the ER leader of the glycoprotein, whereas the GP276 epitope may originate from both, the GP-C precursor protein, or GP2. As shown in Fig. 5*A*, the disappearance of GP1, GP2, and GP-C over time was slowed down by 10 and 80 μM lactacystin to a comparable extent, whereas 3 μM had less and 1 μM had barely any effect. At 80 μM lactacystin the half-life of GP-C was prolonged from about 6 to about 12 h, but the disintegration of glycoproteins was not prevented completely, indicating that in addition to proteasomal degradation, catabolism by other proteases or secretion of the glycoprotein from the cell surface may occur. Interestingly, the ratio of GP2 to GP-C was doubled at a chase period of 36 h at 10 and 80 μM lactacystin, indicating that the GP2 protein is more prone to be stabilized by proteasome inhibition than the GP-C precursor. Our experiments strongly suggest that the reduction in the presentation of GP33 at 0.5 and 1 μM lactacystin cannot be attributed to a decrease in glycoprotein degradation, but may be due to an alteration in peptide processing. At a concentration of 3 μM lactacystin, however, the reduction in GP33 and GP276 degradation may be partially due to a delay in glycoprotein degradation by the proteasome. Also, for the MCMV-pp89 protein we had previously observed a stabilization of the protein at 10 μM lactacystin (41), which was not apparent at a concentration of 1 μM lactacystin (data not shown). To compare the effect of lactacystin on the degradation of LCMV-GP with that of bulk ubiquitin conjugates, we performed a ubiquitin Western blot on total lysates of LCMV-infected MC57 cells treated for 16 h with the indicated concentrations of lactacystin. The result, shown in Fig. 5*C*, indicates that there was no accumulation of high m.w. ubiquitin conjugates at 0.5 μM lactacystin, whereas at 3 μM bulk ubiquitin conjugates were almost as prominent as found for 7 and 10 μM lactacystin. It therefore appears that the degradation of both LCMV-

GP and bulk ubiquitin conjugates was inhibited by lactacystin, with a threshold concentration of about 3 μM.

In contrast to LCMV-GP, the LCMV nucleoprotein turned out to be an extremely stable protein for which we were unable to detect significant degradation in infected B8 fibroblasts even within a chase period of 72 h in the absence or the presence of 3 or 10 μM lactacystin (Fig. 6). An accumulation of the nucleoprotein was found after 3 days of treatment with 10 μM lactacystin, but as the cells suffered considerably from the prolonged treatment, this particular result must be viewed with caution. Clearly, there was no correlation of nucleoprotein degradation and the reduction of NP118 presentation under lactacystin treatment (Fig. 3*A*), suggesting again that the reduction of Ag presentation at 1 μM may be due to an alteration in the cleaving properties of the proteasome.

Low concentrations of lactacystin markedly alter the in vitro fragmentation of polypeptides by the 20S proteasome

We investigated whether a partial and selective inhibition of the proteasomal chymotrypsin-like activity would change the profile of peptide fragments produced by the 20S proteasome in vitro. To this aim we incubated synthetic 25-mer polypeptides containing the GP276 epitope (residues 271–295), the NP118 epitope (residues 108–132), and the MCMV-pp89 epitope (residues 162–186) with purified 20S proteasomes from B8 cells in the presence of 0, 1, and 7 μM lactacystin. A kinetic analysis revealed that the fragments produced did not vary over time under the same conditions up to 24 h when most of the 25 mer was digested. Hence, a 24-h incubation was chosen for analysis by HPLC and Edman degradation. Lactacystin at a concentration of 1 μM slowed the degradation of the 25 mer and altered the quantity and profile of the peptide fragments produced. As an example we show the HPLC profile of the digests of the GP276 containing polypeptide in Fig. 7 (see, for instance, the peak labeled A in Fig. 7). This effect was more pronounced at a concentration of 7 μM lactacystin, and at a concentration of 100 μM lactacystin the peptide fragmentation by the proteasome was completely abolished (not shown). We analyzed the peak that migrated at the same position as the synthetic GP276 11-mer epitope by Edman degradation. The results listed in Table I show that the 11-mer epitope (GP276–286) is a minor peptide in this peak, which also contained 35-fold more of the 12-mer (GP275–286) and 9.4-fold more of the 14-mer (GP273–286) peptide. Although we did not find an absolute enhancement of the amounts of the 11 and 12 mer after treatment with 1.0 μM lactacystin, their amounts relative to the 14 mer increased considerably. These peptides are produced by a cleavage at the same C-terminal residue, but the 14 mer requires cleavage after a leucine residue at the N-terminus compared with an aspartic acid for the 12 mer and a serine for the 11 mer. Cleavage C-terminal of leucine, however, is expected to be preferentially blocked by lactacystin, thus providing a rational for the reduction in 14 mer generation. Taken together, our in vitro analysis provided evidence that the relative quantities of fragments produced by the proteasome is altered in the presence of 1.0 μM lactacystin. These results are consistent with the idea that the altered preferences of proteasome cleavages account for the epitope specific up- or down-regulation of Ag presentation.

Low concentrations of lactacystin are insufficient to induce apoptosis or proliferative arrest

To test whether immunomodulation by lactacystin at 0.5–1 μM would interfere with cellular proliferation, we treated RMA T-cells and MC57 fibroblasts for 3 days with different concentrations of lactacystin and measured the incorporation of tritiated thymidine into newly synthesized DNA every 24 h (Fig. 8). In RMA cells lactacystin did not affect thymidine incorporation at 0.5 or 1 μM lactacystin, whereas in MC57 cells a retardation of proliferation was observed at 1 μM, but not at 0.5 μM, lactacystin. A concentration of 10 μM lactacystin, in contrast, severely interfered with DNA neosynthesis in both cell types. Also, cellular proliferation and viability were reduced at 10 μM, but not at 0.5 or 1 μM lactacystin, as

determined by trypan blue exclusion staining and cell counts over a period of 3 days (not shown).

As lactacystin has been shown to induce cellular differentiation and apoptosis, we determined the concentration of lactacystin required for induction of apoptosis. As an early detection system for apoptosis we used flow cytometric detection of apoptotic cells stained with annexin V-FITC. This method is based on the observation that cells that have received an apoptotic stimulus translocate phosphatidylserine from the inner to the outer leaflet of the plasma membrane, where it is bound by annexin V. In accordance with our proliferation analysis a lactacystin concentration of 0.5 or 1 μM was insufficient to induce apoptosis, whereas an increasing number of annexin V-positive cells became apparent when the lactacystin concentration was raised to 3, 7, or 10 μM lactacystin (Fig. 9). Taken together it appears that low concentrations of lactacystin that partially inhibit the chymotrypsin-like activity of the proteasome can modulate Ag presentation without exhibiting marked adverse effects on cellular proliferation and survival.

Discussion

In this study we investigated how selective inhibitors of the proteasome would affect MHC class I-restricted Ag presentation at different concentrations and whether there is a correlation with the rate of catabolism of the respective proteins. To our surprise we found that at low concentrations of lactacystin and epoxomicin the presentation of the epitopes LCMV-GP33, LCMV-NP118, and MCMV-pp89–168 was reduced, whereas the presentation of the epitope LCMV-GP276 was enhanced. Our data from proteasome in vitro digests suggest that low concentrations of selective proteasome inhibitors suffice to alter polypeptide fragmentation through the proteasome. Neither a prolongation of protein degradation nor an adverse effect on cellular proliferation or viability was observed at these low concentrations, indicating that a pharmacologic modulation of Ag presentation by selective proteasome inhibitors in vivo is feasible.

Recently, we have found that the treatment of LCMV-infected mice with the HIV-1 protease inhibitor Ritonavir at therapeutic concentrations markedly inhibited the generation and expansion of LCMV-specific CTLs in vivo (42). This immunomodulatory effect was neither due to the inhibition of viral replication nor to a direct effect on CTL activation, but was attributed to a reduction in the presentation of immunodominant LCMV epitopes. Interestingly, Ritonavir was found to inhibit the chymotrypsin-like activity of the 20S proteasome in vitro, while the trypsin-like activity was enhanced (41,42). We reasoned that the modulation of proteasome activity would account for the in vivo reduction of Ag presentation, but we could not exclude that this effect was, at least in part, due to the inhibition of other proteases in APC. Hence we decided to investigate the phenomenon of immunomodulation through partial proteasome inhibition with two well-characterized proteasome inhibitors in this study. Lactacystin has been used for several years to study the in vivo effects of proteasome inhibition because it was believed to be exclusively specific for the proteasome (43). The conclusiveness of these experiments must now be questioned, since recent evidence showed that a cathepsin A-like enzyme from lysosomes (44) and tripeptidyl peptidase II (18) were also inhibited by lactacystin. However, at a concentration of 1 μM, which was used for modulation of Ag presentation in this study, tripeptidyl peptidase II is not inhibited, and even at 10 μM lactacystin the inhibition is only 50%. Moreover, lysosomal protein degradation was shown not to affect MHC class I-restricted Ag presentation, suggesting that the modulation of Ag presentation at 1 μM lactacystin is exclusively due to partial inhibition of the proteasome. This idea is corroborated by the observation that epoxomicin had the same effect on the presentation of the epitopes GP33- and GP276 at 100-fold lower concentrations. The proteasome is the only protease known to be inhibited by epoxomicin concentrations of up to 1 μM, which sufficed

to fully inhibit the chymotrypsinlike activity of the proteasome as well as the presentation of GP33 and GP276. Taken together, the data obtained with these two highly specific proteasome inhibitors is very strong evidence that the generation of the four epitopes studied in this work is proteasome dependent and virtually ruled out that the observed effects on Ag presentation are due to the inhibition of other proteases. Moreover, it is reassuring to find that two proteasome inhibitors with the same inhibition profile affect Ag presentation in the same manner, which has not been previously demonstrated.

Lactacystin has the major disadvantage that it is difficult to synthesize and therefore very expensive (45). The synthesis of epoxomicin is comparatively less demanding (36), and as it is effective at much lower concentrations it will be feasible and very interesting to study its effect on the CTL response in LCMV-infected mice. These experiments should also clarify whether the effects on Ag presentation as we have obtained in LCMV-infected fibroblast lines can be extrapolated onto CTL generation and the hierarchy of LCMV epitopes in the infected mouse. In this respect it is interesting that a suppression of the T cell-mediated delayed-type hypersensitivity reaction to picrylchloride by epoxomicin was recently demonstrated in mice (24).

Although we have shown that a partial inhibition of the chymotrypsin-like activity of the proteasome at nontoxic doses may be used to modulated Ag presentation it may be even more attractive to use specific inhibitors of the proteasomal trypsin-like activity or the PGPH activity for this purpose because these agents are likely to have fewer side effects and may be used at higher dosage. From mutagenesis experiments in both yeast and mammalian cell lines it appears that the chymotrypsin-like activity of the proteasome is the most essential for overall protein degradation and cell viability (6–8). For instance, complete replacement of subunit δ, which is in charge of the proteasomal PGPH activity, has been shown to modulate Ag presentation without affecting cellular proliferation or viability (7). Because many T cell epitopes contain glutamic acid or lysines, an inhibition of the PGPH or trypsin-like activity is expected to markedly affect polypeptide fragmentation and Ag processing. The C-terminal anchor residue for most human and all mouse MHC class I molecules is hydrophobic, and therefore the inhibition of the chymotrypsin-like activity is likely to negatively affect the generation of most class I ligands. Unfortunately, proteasome inhibitors that are specific for either PGPH activity or trypsin-like activity are presently not available, but the attempt to produce peptide inhibitors with basic or acidic amino acids in the P1 position and reactive C-terminal groups such as vinyl sulfones, boronic acids, or epoxyketones may result in the development of such inhibitors (46).

The question of whether a higher degradation rate of a protein results in better presentation of epitopes derived from this protein has been investigated in a number of systems. It is unclear at present why accelerated protein degradation led to a better Ag presentation of some epitopes (47–49), while the presentation of others remained unchanged (47,50). For the LCMV glycoprotein and the MCMV pp89 protein it was apparent from our experiments that the extent to which protein degradation was retarded at 3 and 10 μM lactacystin correlated with the reduction of Ag presentation. A very different picture emerged for the LCMV nucleoprotein, which was extremely stable. Even over a chase period of 72 h we failed to observe a significant degradation in either the presence or the absence of lactacystin. Despite its long half-life the nucleoprotein gives rise to the NP118 epitope, which is lactacystin dependent and dominates the H-2L^d-restricted immune response. How can these findings be reconciled? Although the proteasome was shown to be in charge of degrading both short-lived and long-lived proteins (10), the amount of mature protein degraded per time unit must be rather low. One solution to this dilemma has been suggested by Yewdell et al. (51), who proposed the defective ribosomal products hypothesis. According to this hypothesis antigenic peptides from long-lived proteins are not derived from mature and properly folded proteins, but originate from defective

translational products that may be degraded very rapidly. It would be interesting to test this hypothesis for the LCMV nucleoprotein experimentally. Evidence that an enhanced degradation of the LCMV nucleoprotein can promote NP118 presentation has recently been obtained by Rodriguez et al., who showed that a rapidly degraded chimeric protein consisting of a modified ubiquitin and the LCMV nucleoprotein gave rise to more NP118 epitopes than the long-lived nucleoprotein in its wild-type form (49).

The most surprising finding of this study was that, depending on the epitope, low concentrations of lactacystin or epoxomicin can either enhance or reduce Ag presentation. Our pulse-chase experiments performed for the LCMV glycoprotein and nucleoprotein as well as the MCMV pp89 protein provided no evidence that a reduction in protein degradation can account for this phenomenon observed at 0.5 and 1 μM lactacystin. The most likely explanation for this phenomenon is that a selective inhibition of the chymotrypsin-like activity of the proteasome changes the cleavage priorities of the proteasome, leading to the production of different peptide fragments. This conclusion is also consistent with the recent finding that marked allelic differences exist in the extent to which lactacystin affects the stability of different human MHC class I proteins, which is dependent on the availability of appropriate peptide ligands (52). Also, our in vitro data support this conclusion. The digests of three 25-mer polypeptides by purified 20S proteasomes show unambiguously that a 1-μM concentration of lactacystin is sufficient to induce marked changes in the proteasomal fragmentation. This effect becomes even more apparent when higher concentrations of lactacystin are used, but starting with 3 μM lactacystin, protein degradation becomes limiting in vitro and in vivo. These two effects, a better fragmentation at low inhibitor concentrations vs limiting proteolysis at higher concentrations, most likely account for the presentation profile observed for the GP276 epitope, which was maximal at 0.5 μM lactacystin. In vitro, degradation of the LCMV-GP271–295 polypeptide is already slowed at 1 μM lactacystin. However, the fact that cells can proliferate at this same concentration of lactacystin indicates that the protein-degrading capacity of the proteasome in vivo is not yet inhibited to an extent that would limit its housekeeping functions. This makes sense, because the capacity of the proteasome must be high enough to cope with situations of cell stress when much greater amounts of misfolded proteins need to be removed. Taken together, it appears that for generation of the GP276 epitope the cleavage priority of the proteasome is suboptimal rather than the overall capacity to degrade polypeptides, and it is interesting to note in this respect that it is the cleavage priority of the proteasome that is altered by the incorporation of the IFN-γ-inducible subunits LMP2, LMP7, and MECL-1 into the complex (4).

In conclusion, our results strongly suggest that there is a concentration window for the application of selective proteasome inhibitors in which epitope production by the proteasome can be altered without affecting cellular viability. As the cytotoxic immune response tends to focus on a few immunodominant epitopes, it may be feasible to ameliorate CTL-mediated autoimmune assaults by altering epitope generation through the administration of selective proteasome inhibitors.

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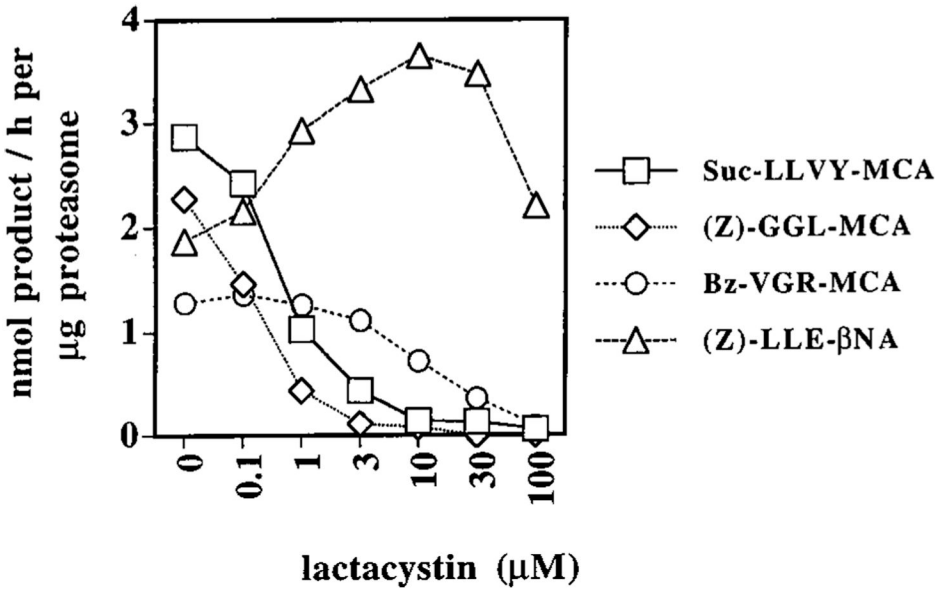

FIGURE 1.

Influence of lactacystin on the hydrolysis of four fluorogenic peptides by the 20S proteasome. Purified 20S proteasomes from B8 fibroblast cells were incubated with one of the substrates and lactacystin at the indicated concentrations for 60 min, when the fluorescence of the fluorogenic leaving group was measured in the linear course of the peptidolytic reaction. Fluorogenic substrates were used at the following concentrations: 100 µM Suc-LLVY-MCA, 100 µM (Z)-GGL-MCA, 400 µM Bz-VGR-MCA, and 200 µM (Z)-LLE-βNA. The displayed values are the means of triplicates with SE of <5% for all data points.

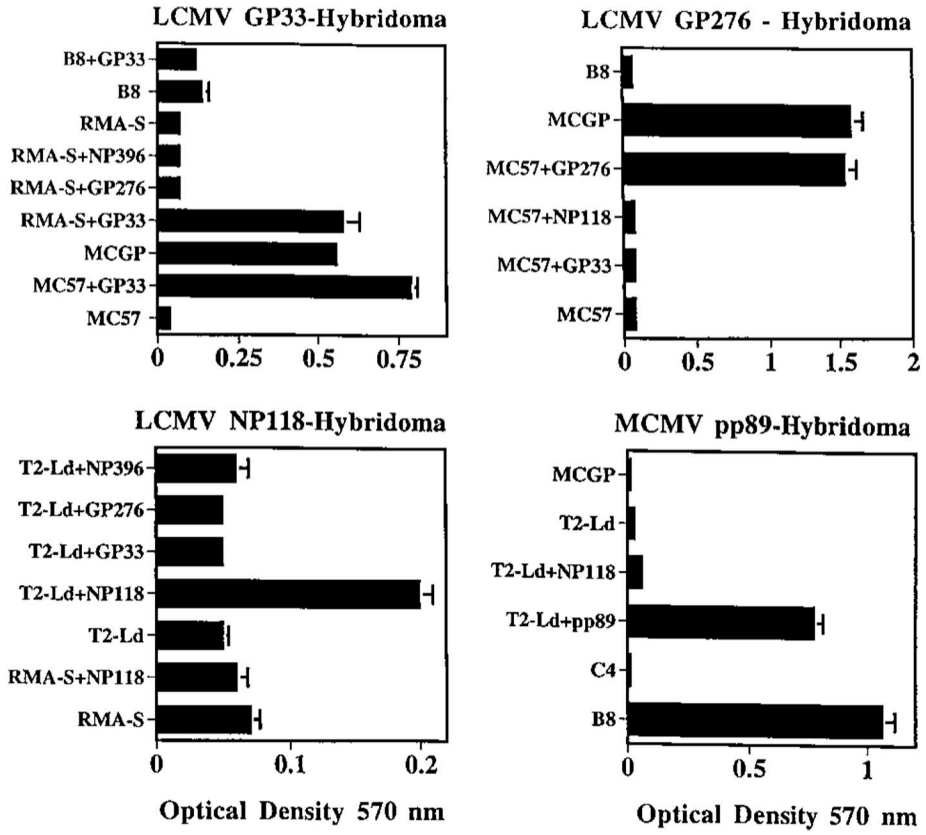

FIGURE 2.

Specificity analysis of T cell hybridomas. The indicated cell lines were used as stimulators either directly or after loading with one of the synthetic peptide epitopes, LCMV-GP33, LCMV-GP276, LCMV-NP396, and MCMV-pp89, at a concentration of 10^{-7} M. The stimulator cells are plotted against the absorbance of the *lacZ* substrate at 570 nm indicative of the TCR-mediated stimulation of the hybridomas. The specificity of the CTL lines that have been used to generate the respective hybridomas is indicated above each panel. B8 is a pp89 transfectant of clone C4, MCGP is a LCMV glycoprotein transfectant of MC57.

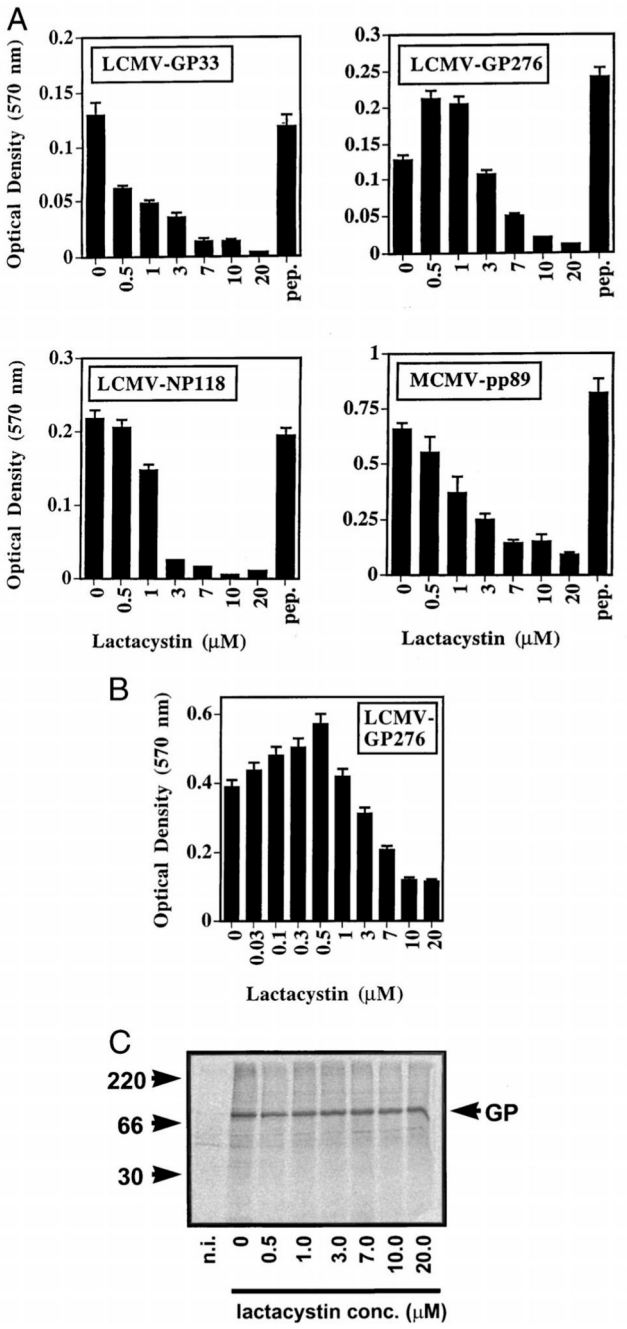

FIGURE 3.

The effects of titrated amounts of lactacystin on the presentation of four different epitopes. *A*, Stimulation of T cell hybridomas of the indicated specificities in chromogenic *lacZ* assays as described in Materials and Methods. Targets used for presentation in the absence or the presence of the indicated concentrations of lactacystin were LCMV-infected MC57 fibroblasts (GP33, GP276), LCMV-infected B8 fibroblasts (NP118), or uninfected B8 fibroblasts (pp89). The B8 clone constitutively expresses low levels of the pp89 protein. To determine maximal stimulation, the uninfected MC57 or B8 cells were externally loaded with the respective synthetic peptides (pep.). The experiment shown is representative of three independent experiments with the same outcome. *B*, Same experiment as in *A* for the GP276 epitope with

further titration of low concentrations of lactacystin. *C*, Immunoprecipitation of LCMV glycoprotein from MC57 cells that were infected with LCMV for 48 h and treated with the indicated concentrations of lactacystin for 24 h before ^{35}S-metabolic labeling for 1.5 h.

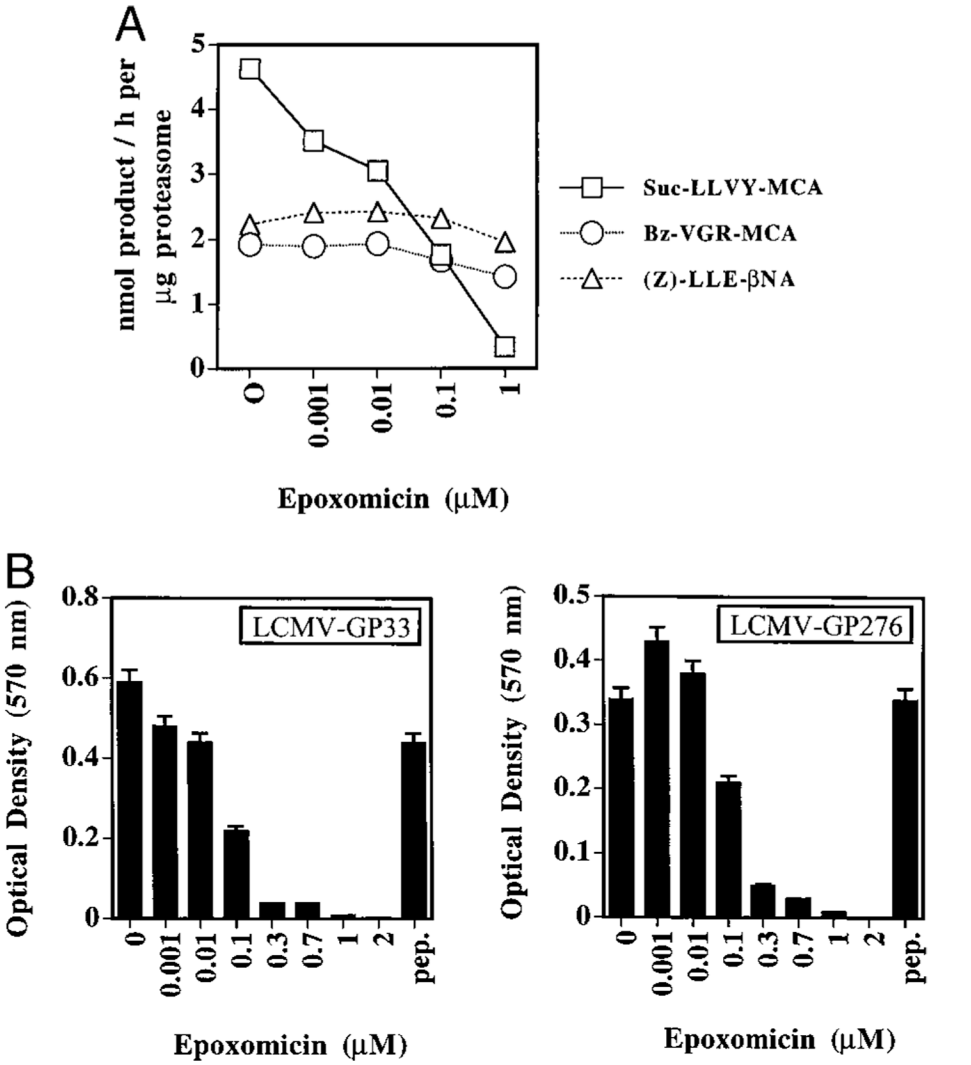

FIGURE 4.

Effect of epoxomicin on peptide hydrolysis by the proteasome and on the presentation of the epitopes GP33 and GP276. *A*, Hydrolysis of the substrates Suc-LLVY-MCA, Bz-VGR-MCA, and (Z)-LLE-βNA by B8 proteasomes in the presence of the indicated concentrations of epoxomicin. The assay conditions were described in Fig. 1. *B*, Presentation of the LCMV epitopes GP33 and GP276 in the presence of the indicated concentrations of epoxomicin. The *lacZ* assay was performed as described in Fig. 3.

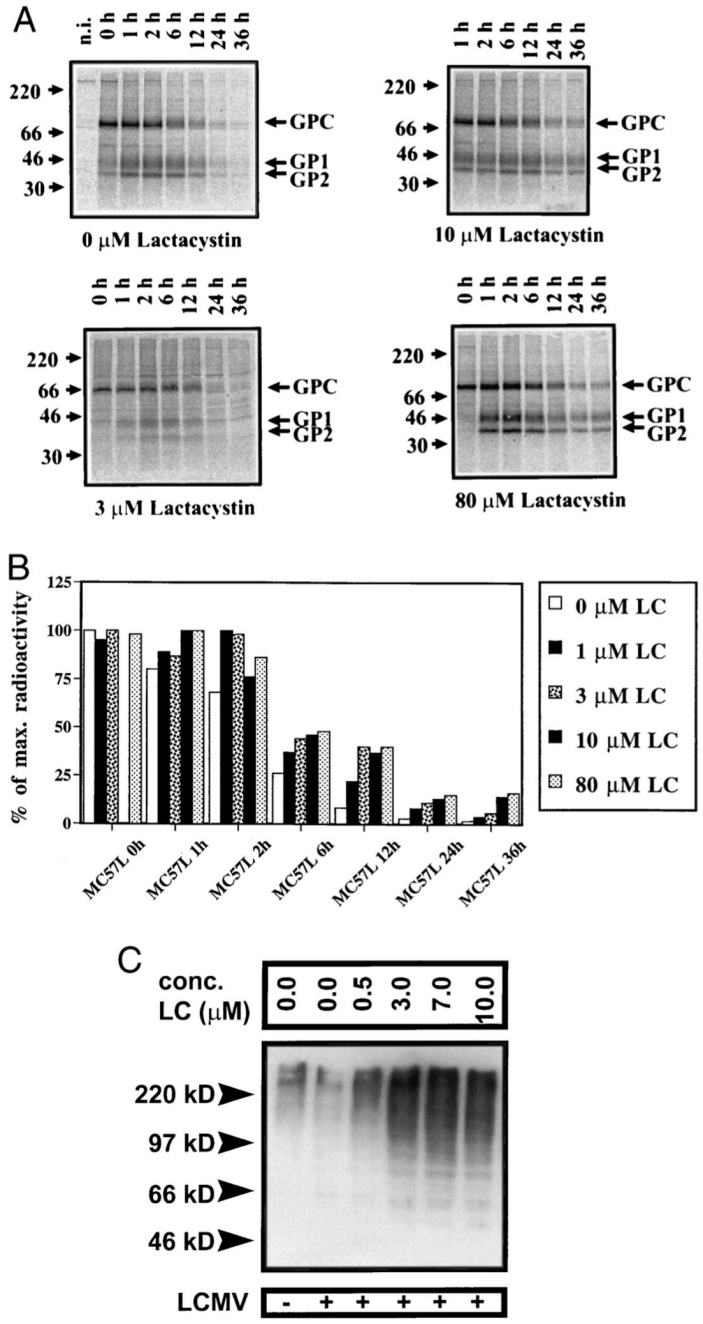

FIGURE 5.

Effect of lactacystin on the degradation of the LCMV gly-coprotein and accumulation of ubiquitin conjugates. LCMV-infected MC57 cells were pulse-labeled with [^{35}S]Met/Cys for 1.5 h, and the cells were chased for the indicated time periods in the presence of 0, 3, 10, or 80 μM lactacystin. *A*, Image of the immunoprecipitated glycoproteins after SDS-PAGE and exposure to a phosphorimager plate. The positions of the glycoprotein precursor (GPC, 70 kDa) and the GP1 (44 kDa) and GP2 (35 kDa) glycoproteins are indicated. As a negative control (n.i.), uninfected MC57 cells were used. *B*, Graphic representation of a quantitative analysis of radioactivity in immunoprecipitated GPC. *C*, Ubiquitin Western blot of total lysates from LCMV-infected MC57 cells treated for 16 h with the indicated concentrations of lactacystin.

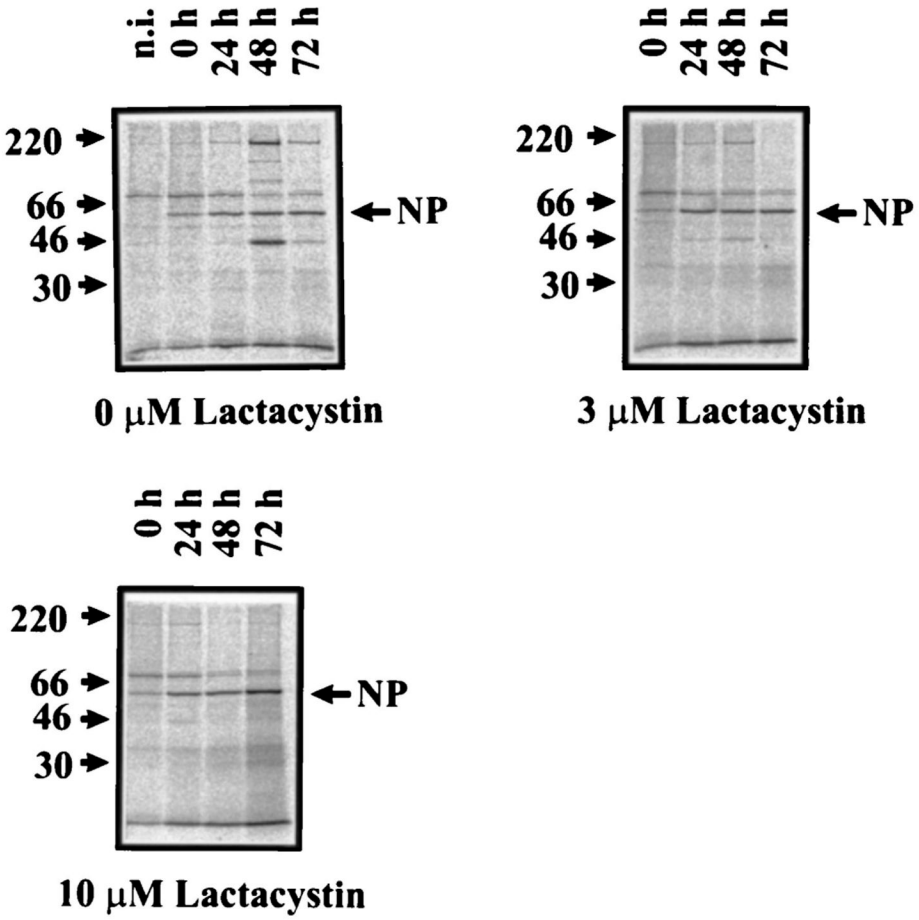

FIGURE 6.

The effect of lactacystin on degradation of the LCMV nucleoprotein. LCMV-infected B8 cells were pulse labeled with [^{35}S]Met/ Cys for 1.5 h, and the cells were chased for the indicated time periods in the presence of 0, 3, or 10 μM lactacystin. An image of the immunoprecipitated nucleoproteins after SDS-PAGE and exposure to a phosphorimager plate is shown. The position of the 63-kDa LCMV nucleoprotein (NP) is labeled. As a negative control (n.i.), uninfected B8 cells were used.

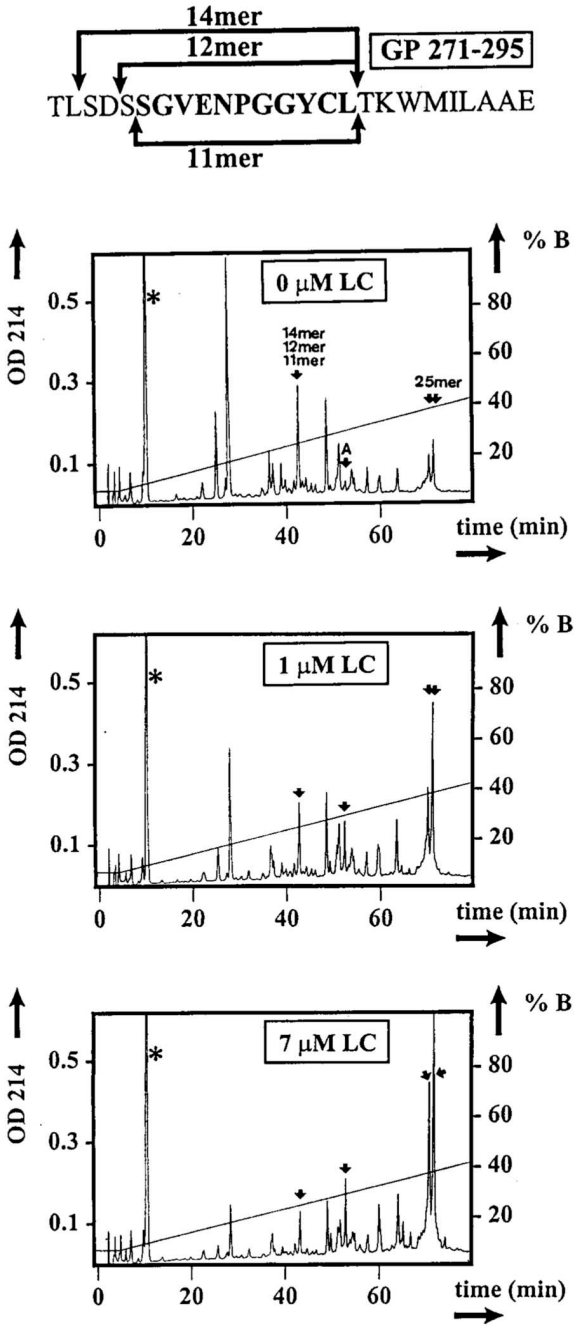

FIGURE 7.

HPLC profiles of proteasome digests of an LCMV GP271–295 synthetic 25 mer peptide in the presence of 0, 1, and 7 μM lactacystin (LC). The digests were performed with 20S proteasomes purified from B8 fibroblasts for 24 h. The peak labeled 11 mer/12 mer/14 mer was chosen for analysis by Edman degradation (Table I) because it migrates at the same position as the 11-residue-long synthetic T cell epitope GP276–286. Peak A was labeled to illustrate the marked differences in the profile due to inhibition with 1 and 7 μM lactacystin. The peak labeled with an asterisk is due to DTT in the reaction buffer.

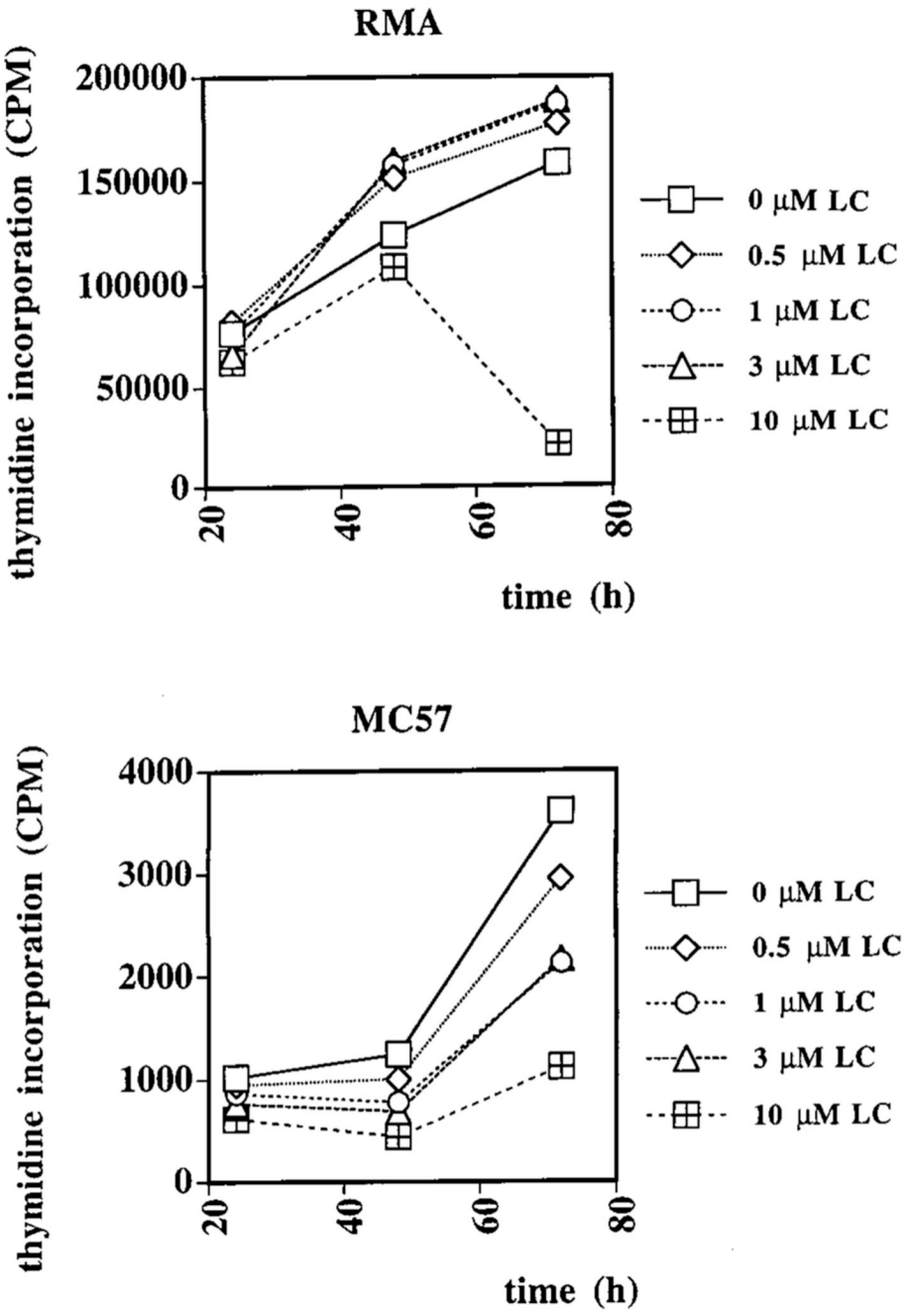

FIGURE 8.

The impact of different concentrations of lactacystin on thymidine incorporation of RMA T-cells and MC57 fibroblasts. The incorporation of [^{3}H]thymidine in cellular DNA during a 16-h pulse was measured after 24, 48, and 72 h of cultivation in the presence of the indicated concentrations of lactacystin. Values represent the means of triplicates with SEs of <5% for all data points.

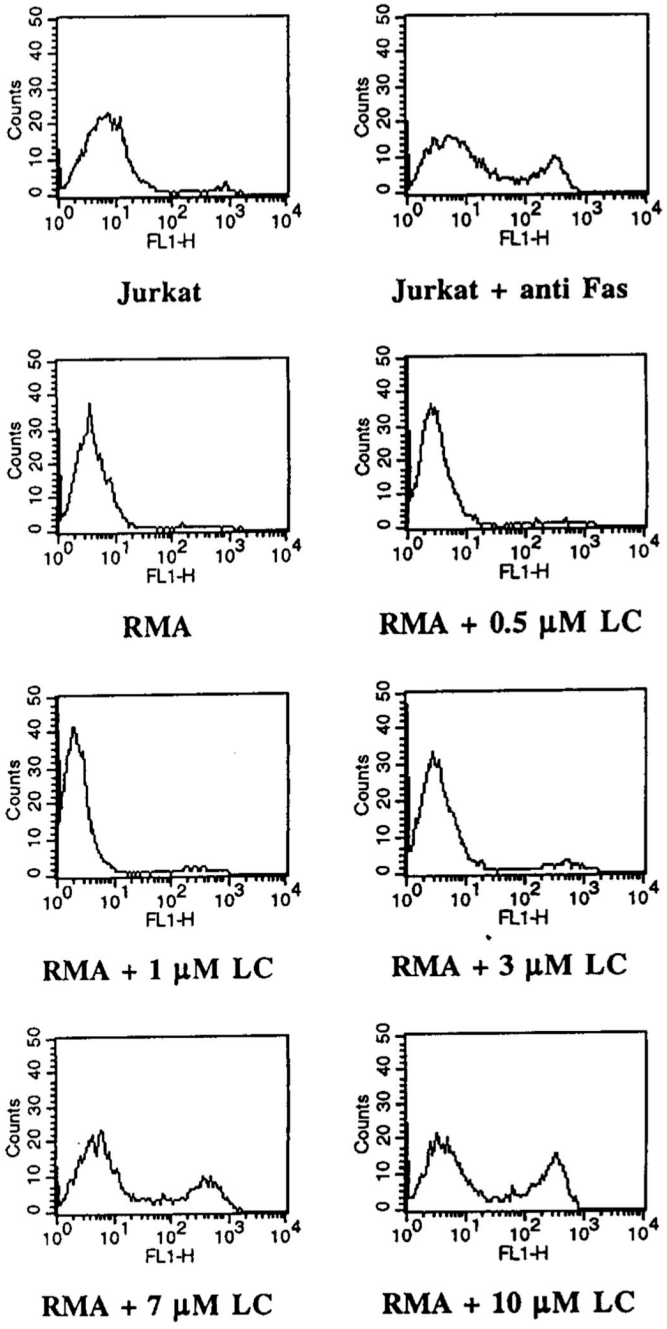

FIGURE 9.

Apoptosis induction by lactacystin treatment at different concentrations. RMA cells were incubated for 14 h with the indicated concentrations of lactacystin before surface staining with annexin V-FITC and flow cytometric analysis of viable cells. As positive and negative controls, Jurkat cells were incubated for 6 h in the presence and the absence of an anti-human FAS mAb, respectively.

Table I

Results of Edman degradation of selected peptides produced from 25-mer polypeptide LCMV GP271–295

LC Concentration (μM)	GP276–286 11-mer pmol[a]	GP275–286 12-mer pmol[a]	GP273–286 14-mer pmol[a]
0	1.13 (100%)	40.3 (100%)	10.6 (100%)
1	0.96 (85%)	34.3 (85%)	5.8 (55%)
7	0.76 (67%)	21.6 (54%)	2.7 (25%)

[a]The values represent averages of yields of phenylthiohydantoin derivatives from three selected amino acids that could be unambiguously assigned to one of the peptides GP276–286, GP275–286, or GP273–286. These three peptides were found in a single fraction labeled 11-mer/12-mer/14-mer in the HPLC profile shown in Fig. 7.